IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	**09/932,521**
Applicants	:	**Hans Herweijer**
Filed	:	**08/17/2001**
Art Unit	:	**1632**
Examiner	:	**Woitach, Joseph T.**
Docket No.	:	**Mirus.023.01**
For	:	**Nucleic Acid Expression From Linear Nucleic Acids**

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as express mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on this date: 5/19/2006.

Mark K Johnson

APPELLANT'S BRIEF

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1. Real party in interest:

The real parties in interest are: Hans Herweijer and Christine I. Wooddell and, by assignment, Mirus Corporation, which has changed its name to Mirus Bio Corporation under the laws of the State of Delaware and is located at 505 South Rosa Road, Madison, WI 53719.

2. Related appeals and interferences:

There are no interferences known to appellant, the appellant's legal representative, or assignee which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

3. Status of Claims:

Claims 1-15 and 17 have been rejected and are hereby appealed.

Claims 16 and 18-20 have been canceled.

4. Status of amendments:

No Amendments have been filed subsequent to the rejection.

5. Summary of claimed subject matter:

The claimed subject matter is a process for improving expression of a transgene *in vivo*. It has frequently been found that the persistence of expression of a heterologous gene delivered non-virally to a cell *in vivo* is relatively short. The standard practice in the art is to isolate DNA from bacteria in the form of closed circular supercoiled plasmids. The plasmid DNA is then directly delivered to the cell in vivo. Expression from such plasmid DNA can be substantially reduced in as little as one day. Applicants have discovered that, by linearizing the plasmid prior to delivery to cells, expression of the delivered gene persists for a substantially longer period of time. The preferred methods for linearization of the plasmid, restriction enzyme digestion and polymerase chain reaction, are well known in the art.

Covalently closed circular plasmid DNA was thought to be more stable *in vivo*, since exonucleases, which digest DNA from terminal ends, would not have access to a circular plasmid. However, Applicants have shown detectable expression from a transgene delivered as a linear DNA for as long as 182 days. The same transgene, when delivered as a closed circular supercoiled plasmid, was undetectable after 28 days (specification page 19, line 18). Increased expression from linear DNA presents at least two important improvements over the standard process of circular plasmid gene delivery *in vivo*. The first advantage is the obvious benefit of improved expression over a longer period of time. Second, because linear DNA is used, bacterial sequences, which must be present in plasmid DNA for its production and has been shown to be immunogenic, can be eliminated. Removal of such sequences reduces toxicity and immune-related suppression of transgene expression.

Human factor IX (ng/ml) was measured in the plasma averaged from mice as shown in the following table (specification page 23, line 7).

	Human factor IX expression level in mouse plasma (in ng/ml) at various days after pDNA injection			
Injected DNA	**Day 1**	**Day 7**	**Day 28**	**Day 62**
Supercoiled plasmid	55,620	9	< 1	<1
Blunt-linearized plasmid	48,169	119	137	385

6. Grounds of rejection to be reviewed on appeal:

Whether claims 1-15 and 17 are unpatentable under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed has possession of the claims invention.

Whether claims 1-15 and 17 are unpatentable under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

7. Argument:

Rejection of claims 1-15 and 17 under 35 U.S.C. 112, first paragraph: The Action states, on page 3, that there is a lack of specific teaching for the invention as claimed and that there is not adequate guidance to practicing the breadth of the claims. The Action states

> "... *the literal support for the terminology is acknowledged, however the specification contemplates the use of a variety of promoter types including conditional promoters which would not necessarily be active at injection or at seven days. The basis of maintaining the rejection is that the claims are drawn to specific embodiments set forth in the specification which were not specifically contemplated together.*"

The Action goes on to state that expression levels will depend on the promoter, means of delivery, and stability of the gene product itself.

Applicants do not dispute that these factors will affect the overall level of gene expression. However, choosing an appropriate promoter is a matter of experience and preference that is well known by one having skill in the art as well as choosing the means of delivery. The stability of the gene product is provided in claim 1 as "*increased expression in the hepatocyte after seven days defined by at least 20% more gene product than is expressed from a supercoiled plasmid from which the linearized plasmid is derived.*"

Applicants have shown that expression of a DNA sequence persists for a longer time and at higher levels if the expression cassette is delivered to the cell *in vivo* as a linear DNA rather than the same sequence in the known delivery vehicle, a covalently closed circular plasmid.

The Action notes that the specification must teach those of skill in the art how to make and how to use the invention as broadly claimed. Applicants believe this requirement has been met. Applicants have taught that the injection of naked plasmid DNA (pDNA) into liver or tail vein vessels leads to high levels of foreign gene expression (specification page 4, line 1). Efficient methods for delivering plasmid

DNA is described in the specification beginning on page 4, line 25 in the section entitled Delivery of Nucleic Acids (references provided in the evidence appendix).

One with skill in the art must provide the gene to be delivered in the form of a linear DNA rather than an art-standard circular plasmid. The means for forming a linear DNA are described in the specification on page 7, beginning on line 13 in the section entitled Linear DNA. Other methods are well established in the art.

Therefore, Applicants believe that they have provided sufficient support in the specification to show that the new matter rejection is groundless and to enable one skilled in the art to make and/or use the invention.

Rejection of the claims 1-15 and 17 under 35 U.S.C. 112, second paragraph: The Action states that the claims are indefinite because there is no embodiment that requires the expression cassette to be active in the liver.

The claims recite delivering the linearized plasmid DNA vector to a hepatocyte, a liver cell, in a mammal. It is well known by one skilled in the art that expression in a hepatocyte requires a promoter that is active in the liver. Such promoters are readily recognized and determined. On page 12, line 32 of the specification in the section entitled Expression Cassette, Applicants state that "A DNA expression cassette typically includes a promoter (allowing transcription initiation)." On page 20, line one, the statement "Expression cassettes that contain sequences to be transcribed in mammalian cells require a promoter of mammalian or viral origin" indicates that one having skill in the art would have knowledge of such promoters.

It is well known that liver active transcription factor/promoters in the art include: viral promoters such as CMV, RSV, hepatitis B, and SV40 promoters, and liver promoters/factors such as albumin promoter, glucokinase promoter, CCAAT/enhancer binding protein, connexin 32 gene B2 element, D-site binding protein, hepatic leukemia factor, hepatic nuclear factor, insulin response element, metal response element, methylated DNA-binding protein site, peroxisome proliferator response element, promoter linked coupling element, xenobiotic response element, alpha-fetoprotein promoter, alpha1-antitrypsin promoter.

The Action also states that "*there is no embodiment within the claims that requires ... functional properties to assess expression levels at a later time.*" Applicants are claiming a process for expression of DNA and do not believe that it is necessary for them to include "*functional properties*" in the context of the claims or why expression assessment is expected to be recited in the claims. Assessment of gene expression is routinely practiced by those skilled in the art. Methods of assessing gene expression practiced in the art include: Northen Blot analysis, ELISA, reverse transcriptase PCR, Western blot analysis, and enzyme assays.

8. Claims Appendix:

1. (previously presented) A process for *in vivo* expression of longer than seven days of a non-viral, linear DNA nucleic acid sequence from a delivered expression cassette, comprising:

   a) providing the expression cassette comprising the nucleic acid sequence operably linked to a promoter;

   b) forming a non-viral, linearized plasmid DNA vector comprising the expression cassette; and,

   c) delivering the non-viral, linearized plasmid DNA vector to a hepatocyte in a mammal, wherein providing the expression cassette on the non-viral, linearized plasmid DNA vector results in increased expression in the hepatocyte after seven days defined by at least 20% more gene product than is expressed from a supercoiled plasmid from which the linearized plasmid is derived.

2. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains blunt ends.

3. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains sticky ends.

4. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains a blunt end and a sticky end.

5. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector is generated by restriction enzyme digestion.

6. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector is generated by polymerase chain reaction.

7. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains an expression cassette isolated from a plasmid backbone.

8. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains an expression cassette which is flanked by sequence derived from inner Tn5 transposase recognition elements.

9. (previously presented) The process of claim 8, wherein the non-viral, linear DNA vector ends are blunt.

10. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains an expression cassette which is flanked by sequence derived from outer Tn5 transposase recognition elements.

11. (previously presented) The process of claim 10, wherein the non-viral, linear DNA vector ends are blunt.

12. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector contains an expression cassette which is flanked by chimeric ends derived from Tn5 transposase recognition elements.

13. (previously presented) The process of claim 12, wherein the non-viral, linear DNA vector ends are blunt.

14. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector is delivered to cells intravascularly.

15. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector are delivered intravascularly using pressure.

16. (canceled)

17. (previously presented) The process of claim 1, wherein the non-viral, linear DNA vector is delivered by direct interstitial injection.

18. (canceled)

19. (canceled)

20. (canceled)

9. Evidence appendix:

Copies of the references cited in the specification beginning on page 4, line 25 from the section entitled Delivery of Nucleic Acids can be found in this appendix. The cited references are:

G. Zhang, V. Budker and J.A. Wolff. Human Gene Therapy 10:1735-1737, 1999.

V. Budker, G. Zhang, S. Knechtle and J.A. Wolff. Gene Ther. 3:593-598, 1996.

G. Zhang, D. Vargo, V. Budker, N. Armstrong, S. Knechtle and J.A. Wolff. Hum.

HUMAN GENE THERAPY 10:1735–1737 (July 1, 1999)
Mary Ann Liebert, Inc.

Brief Report

High Levels of Foreign Gene Expression in Hepatocytes after Tail Vein Injections of Naked Plasmid DNA

GUOFENG ZHANG, VLADIMIR BUDKER, and JON A. WOLFF

ABSTRACT

We have previously shown that the intramuscular injection of naked plasmid DNA enables foreign gene expression in muscle. Further studies showed that the intravascular delivery of naked plasmid DNA enables high levels of expression not only in muscle but also in hepatocytes. For the liver, this technique required injection directly into the liver vessels (portal vein, hepatic vein, or bile duct) and occlusion of outflow. The present study now demonstrates that high levels of plasmid DNA expression in hepatocytes can be easily obtained by tail vein injections. The highest levels of expression are achieved by rapidly injecting the plasmid DNA in large volumes, ~2.5 ml. This technique has great potential for a wide variety of laboratory studies.

INTRODUCTION

THE TRANSFECTION OF PLASMID DNA (pDNA) into mammalian cells is an indispensable tool in molecular and cellular biology. The anticipated avalanche of new genes that will be discovered as part of the human genome project will increase the importance of gene transfer in understanding gene function. Heretofore, the analysis of gene expression in a living mammal has been limited since it has required laborious procedures such as the production of viral vectors or generation of transgenic mice.

We now show that high levels of foreign gene expression can be achieved in hepatocytes after the rapid tail vein injection of naked pDNA in a large volume of physiologic solution. These results extend our previous observations that high levels of expression are achieved by the direct, intravascular administration of naked pDNA into the vessels supplying the liver or muscle (Wolff *et al.*, 1990; Budker *et al.*, 1996, 1998; Zhang *et al.*, 1997; Wolff, 1998).

MATERIALS AND METHODS

Plasmids and reporter gene systems

The pCILuc plasmid expresses a cytoplasmic luciferase from the human cytomegalovirus (CMV) immediate-early promoter (Zhang *et al.*, 1997). The luciferase assays were done as previously reported (Wolff *et al.*, 1990). Relative light units (RLU) were converted to nanograms of luciferase by using standards from Molecular Probes (Eugene, OR). The amount of luciferase was calculated by using a standard curve in which luciferase protein (pg) = $5.1 \times 10^{-5} \times \text{RLU} + 3.683$ ($r^2 = 0.992$).

pCILacZ expresses the *Escherichia coli lacZ* gene from the CMV immediate-early promoter (Zhang *et al.*, 1997). Ten micrometer sections were stained for β-galactosidase expression by 4-hr X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) incubations.

Injection techniques

Male and female ICR mice were used for all experiments except when noted. The direct injections into the liver were done through either the portal vein or hepatic vein (via the inferior vena cava) under optimal conditions for expression as previously reported (Zhang *et al.*, 1997). The optimal conditions entailed the injection of pDNA in 1 ml of normal saline (0.9% NaCl) containing 15% mannitol (Sigma, St. Louis, MO) and heparin (2.5 units/ml; Lypho-Med, Chicago, IL) (Zhang *et al.*, 1997). For the portal vein injections, outflow was blocked by occluding the hepatic vein and inferior vena cava. For the hepatic vein injections, outflow was blocked by occluding the portal vein, vena cava, and hepatic artery.

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The tail vein injections were done by injecting through a 27-gauge needle 10–250 μg of pDNA in 1–3 ml of Ringer's solutions (147 m*M* NaCl, 4 m*M* KCl, 1.13 m*M* $CaCl_2$) over 7–120 sec.

RESULTS AND DISCUSSION

The rapid injection of 100 μg of pCILuc in 2.5 ml of Ringer's solution into the tail vein of ICR mice allowed expression levels similar to those achieved by direct injection into the portal vein or hepatic vein (Fig. 1A). For each liver, the luciferase activities were similar in all the lobes. Maximal luciferase expression by the tail vein approach was achieved when the DNA solution was injected within 7 sec (Fig. 1B). Luciferase expression was also critically dependent on the injection volume (Fig. 1C). A dose–response relationship between luciferase expression and the amount of pCILuc injected was observed and substantial levels of expression were observed with only 10 μg of plasmid (Fig. 1D). Similar levels of expression were obtained with C57/B mice. As in our previous studies using direct liver vascular injections, luciferase expression was transient and had fallen to minimal levels by 1 week after tail vein injection (data not shown).

The β-galactosidase reporter gene system was used to determine the type and percentage of cells transfected. After injecting 100 μg of pCILacZ (an *E. coli* β-galactosidase expression vector) in 2.5 ml within 7 sec, 1–20% ($n = 3$) of the hepatocytes expressed β-galactosidase (Fig. 2). The extent of β-galactosidase staining is consistent with the tail vein luciferase levels and with previous results using direct liver vessel injections. Few of the nonparenchymal cells expressed β-galactosidase.

Direct observation of the liver during tail vein injection indicated that the liver swelled and blanched. The rapid injection of the large volume of fluid enables plasmid delivery to the hepatocytes by causing a transient right-sided congestive heart failure and back-pressure to the liver vessels. This effect is similar to that of direct injection into liver vessels with occlusion of outflow vessels (Zhang *et al.*, 1997). Efficient expression is dependent on raising the intravascular hydrostatic pressure, causing an extravasation of fluid that carriers the pDNA into contact with hepatocytes.

Histologic analysis indicated that liver necrosis was present in about half of the mice by 1 day postinjection. Quantitative evaluation of 16,000 hepatocytes from four livers indicated that 8.2 (±3%) of the hepatocytes were necrotic. Serum alanine aminotransaminase (ALT) increased to a mean of 3480 (±1641, $n = 6$) by 1 day after injection and then returned to near normal levels by 2 days. The small amount of necrosis on histologic analysis is consistent with the limited rise in serum ALT. There was no correlation between necrosis and either the level or location of β-galactosidase expression. This result is in agreement with our previous studies using direct intraportal injection of naked DNA, in which no correlation between necrosis and either the level or location of liver expression was found (Her-

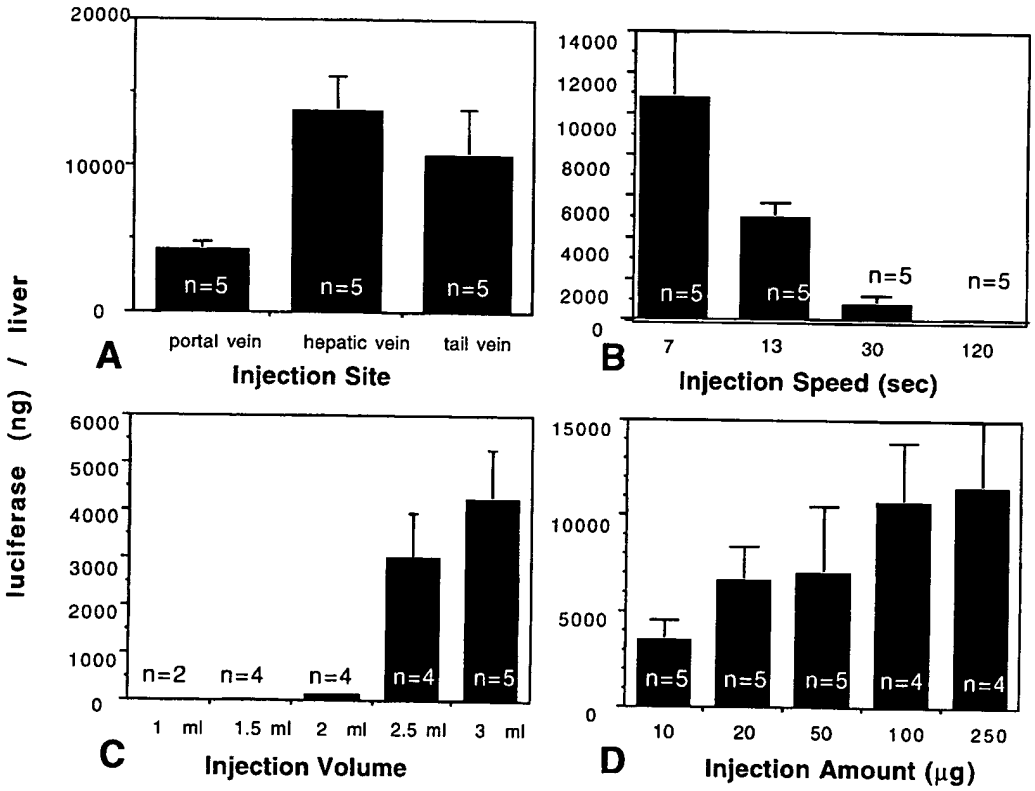

FIG. 1. The effect of injection parameters on luciferase levels one day after injection. *n*, Number of animals.

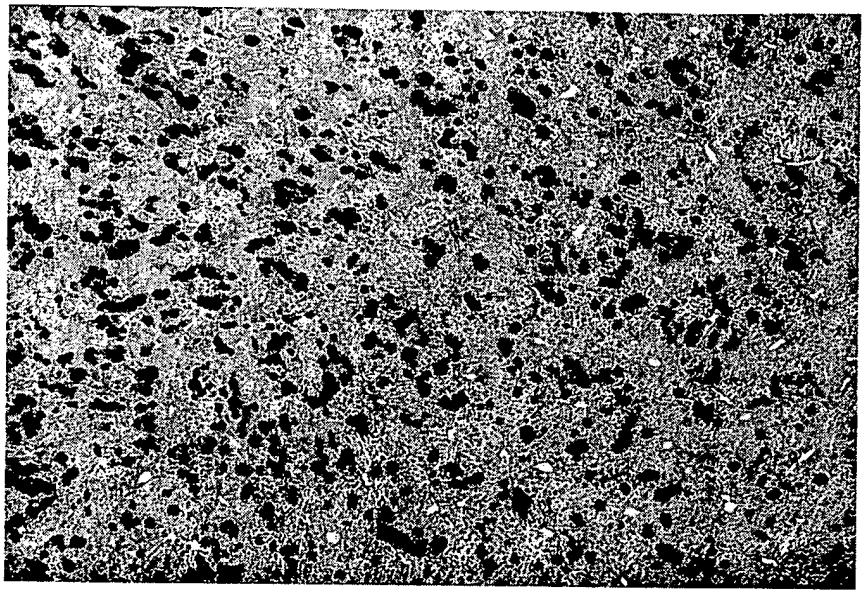

FIG. 2. β-Galactosidase expression 1 day after tail vein injection of 100 μg of pCILacZ. One of the sections with the highest levels of staining is presented.

weijer and Wolff, 1999). The necrosis is most likely due to the increased intravascular pressure in the liver vessels.

Mouse survival after the injection of the large volume was 100%. Of the 51 ICR mice injected (including 41 mice injected with 2.5 or 3 ml), only 1 died as a result of anesthesia. Six C57 mice were also treated without any deaths. The use of normal saline instead of Ringer solution resulted in only a 60% survival rate.

After injection with Ringer's solution, the body weight remained elevated for 30 min and then slowly returned to baseline by 2.5 hr. The 30-min delay in reducing body weight (presumably by urination) could be due to the injected fluid having entered the extravascular space during the rapid injection and then slowly returned to the intravascular space.

Our results with naked DNA need to be taken into account in interpreting the results of the use of pDNA-based, nonviral vectors for targeting hepatocytes after tail vein injection. Some of these protocols use large volumes of injection fluid to prevent the aggregation of the DNA complexes. It is possible that not all of the DNA is complexed or that the DNA dissociates *in vivo* (e.g., from serum protein interactions). Naked DNA should be used as a control and standard for comparison.

In the research laboratory, this robust and facile technique will enable mice to be used just as immortalized and primary liver cell cultures are now used for gene and cellular studies of liver function. A variety of metabolic processes, including secreted and receptor proteins, could also be studied within the context of the whole mammalian organism. Given that the high levels of expression are transient in this system, it would be best if these effects occurred within a few days of treatment (Zhang *et al.*, 1997).

REFERENCES

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Gene Therapy (1996) 3, 593–598

Naked DNA delivered intraportally expresses efficiently in hepatocytes

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Naked plasmid DNA in hypertonic solutions was injected intraportally in mice whose hepatic veins were transiently occluded. High levels of luciferase expression and β-galactosidase expression in 1% of the hepatocytes throughout the entire liver were achieved using 100 μg of the respective plasmid vector two days after the intraportal injection of 1 ml of a solution containing 0.9% saline, 15% mannitol and 2.5 units/ml of heparin. With 100 μg of pCMVGH, the mean hGH serum concentration was 65 ng/ml ± 26 (n = 7), which is approximately 50-fold above normal baseline levels. These unprecedented levels of foreign gene expression from naked plasmid DNA document the ability of parenchymal cells in vivo to take up naked DNA following intravascular delivery.

Keywords: gene transfer; gene therapy; liver; hepatocytes; plasmid DNA; human growth hormone

Introduction

The ability for naked plasmid DNA to be expressed at substantive levels in mammalian cells was first demonstrated in muscle after its intramuscular injection *in vivo*.[1] Subsequently, the expression of naked DNA has been described following the direct interstitial injection of rat liver, melanomas, pig epidermis, and rabbit thyroid as well as lung by intratracheal injection and into arteries using a hydrogel-coated angioplasty balloon.[2–9] However, the levels achieved by the interstitial injection of naked DNA in these tissues would limit their usefulness in the clinical setting.

The liver is an important target tissue for gene therapy by virtue of its central role in metabolism and the production of serum proteins. A variety of techniques have been developed to transfer genes into the liver. Cultured hepatocytes have been genetically modified by retroviral vectors and implanted back into the livers of animals and humans.[10–13] Retroviral vectors have also been delivered directly to livers in which hepatocyte division was induced by partial hepatectomy.[14–16] Injection of adenoviral vectors into the portal or systemic circulatory systems leads to high levels of foreign gene expression that are transient.[17–19] Nonviral transfer methods have included polylysine complexes of asialoglycoproteins that are systemically administered.[20–22] Transient foreign gene expression in limited areas has also been achieved by repetitively injecting naked DNA in isotonic solutions into the liver parenchyma of animals treated with dexamethasone.[8,9] Plasmid DNA expression in the liver has also been achieved via liposomes delivered by tail vein or intraportal routes.[23–25] Nevertheless, there remains a need for an efficient and safe gene transfer method that can either express foreign genes in the liver stably or that can be administered repetitively.

We report that intraportal injection of plasmid DNA in hypertonic solutions and under hydrostatic pressure leads to highly efficient levels of foreign gene expression in hepatocytes throughout the liver. These unprecedented levels of expression from naked DNA have implications for the mechanism of naked DNA uptake and gene therapy.

Results

Requirements for efficient expression

After the livers of 25 g, 6-week-old mice were exposed through a ventral midline incision, 100 μg of pBS.CMVLux plasmid DNA in 1 ml of various solutions was injected into the portal vein via a 30-gauge, 1/2-inch needle over approximately 30 s. Approximately 15% of the mice did not survive the procedure as a result of bleeding. Two days after injection, a mean of only 0.4 ng of total luciferase per liver was produced when the DNA was delivered intraportally in an isotonic solution without ligation of the hepatic vein (Figure 1, condition 1). Inclusion of 20% mannitol in the injection solution increased the mean total luciferase per liver over 10-fold to 4.8 ng (Figure 1, condition 2).

In order to prevent the DNA's rapid transit and to increase the intraportal hydrostatic pressure, the hepatic vein was clamped for 2 min after injection. Luciferase production increased a further three-fold to 14.7 ng (Figure 1, condition 3). When the DNA was injected in a hypertonic solution containing 0.9% saline, 15% mannitol and 2.5 units/ml of heparin to prevent microvascular thrombosis[26] and with the hepatic vein clamped, luciferase expression increased eight-fold to 120.3 ng per liver (Figure 1, condition 5). Luciferase activities in each liver were evenly distributed in six divided sections assayed (data not shown). If the mannitol was omitted under

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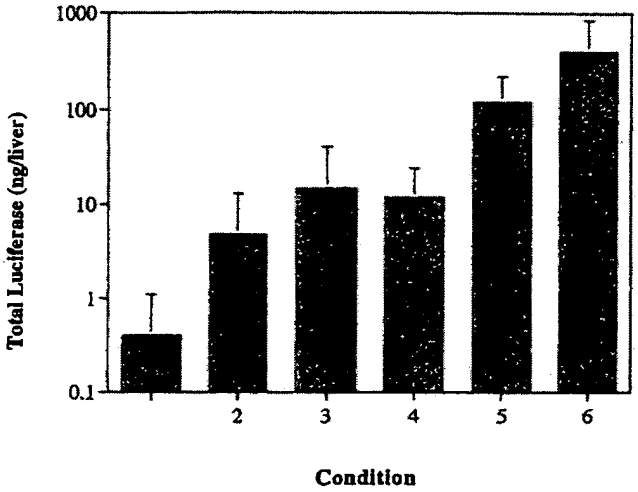

Figure 1 Mean total luciferase in the liver 2 days after the intraportal injection of 100 μg pBS.CMVLux in 1 ml of different solutions with no clamp or with the hepatic vein and inferior vena cava clamped. Lane 1: no clamp, normal saline solution (NSS), n = 6. Lane 2: no clamp, 20% mannitol, n = 3. Lane 3: clamp, 20% mannitol, n = 9. Lane 4: clamp, 2.5 units heparin/ml in NSS, n = 4. Lane 5: clamp, 15% mannitol and 2.5 units heparin/ml in NSS, n = 12. Lane 6: clamp, 15% mannitol and 2.5 units heparin/ml in NSS in animals that received dexamethasone, n = 8. T bars indicate the standard deviation.

these conditions, luciferase expression was 10-fold less (Figure 1, condition 4)). These results indicate that hypertonicity, heparin and hepatic vein closure are required to achieve very high levels of luciferase expression.

The effects of other factors on expression were explored. If the total volume of the injection fluid was 0.5 ml instead of 1.0 ml, luciferase expression decreased 70-fold (data not shown) suggesting that 0.5 ml was not sufficient to fill the intravascular space and distribute the DNA throughout the parenchyma.[27–29] Compared with the results with 100 μg of pBS.CMVLUX, luciferase expression was approximately seven-fold less if 20 μg of pBS.CMVLux DNA was injected, but luciferase expression was not greater with 500 μg of plasmid DNA (data not shown). If the injections were done under the optimal conditions described above (ie hypertonic solution containing heparin and hepatic vein closure) into animals that had been injected with daily injections of dexamethasone starting the day before plasmid injection, luciferase expression was three-fold greater than the expression without dexamethasone (Figure 1, condition 6). The magnitude of this effect is consistent with dexamethasone's activation of the CMV promoter but other cellular effects cannot be excluded.[8,9]

Identification of transfected cell type

Having defined the optimal conditions, the types and percentages of transfected cells were determined. After injections of 100 μg of the cytoplasmic (pBS.CMVLacZ) or the nuclear (pBS.CMVnLacZ) β-galactosidase expression vectors into dexamethasone-treated animals, liver cryosections 10- to 30-μm thick were stained for β-galactosidase using X-gal at pH 7.5 to prevent background staining. Intense blue staining was observed in approximately 1% of the liver cells and was evenly distributed throughout the liver (Figure 2). X-gal incubations for only 1 h resulted in intensely blue cells, suggesting that the

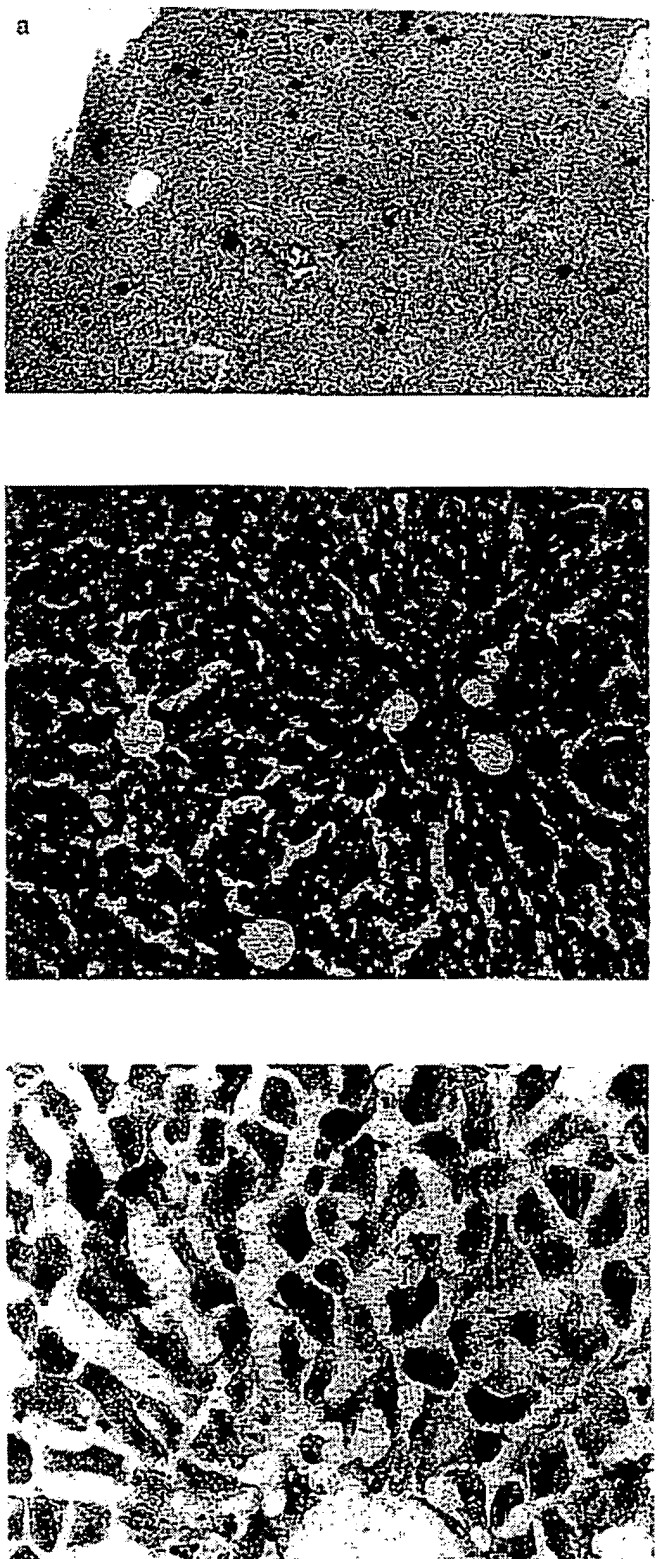

Figure 2 β-Galactosidase staining of livers injected 2 days previously with 100 μg of pBS.CMVLacZ (a and b) or pBS.CMVnLacZ (c) under optimal conditions. X-gal staining was performed for 1 h. (a) 10-μm section, × 100; (b), 30-μm section, × 250; (c), 10-μm section, × 1000; washing step after hematoxylin incubation omitted to decrease its nuclear staining.

grounds. The few small, nonhepatocyte cells with blue staining appeared to be Kupffer and endothelial cells.

Increased osmotic and hydrostatic intraportal pressure was required to achieve high levels of expression. These conditions presumably enhanced DNA transfer to hepatocytes by opening transiently the intraportal endothelial barrier.[32–34] The approximately 0.1 μm size of fenestrae in the sinusoid endothelial cells under normal conditions[27–29] would prevent the exit from the sinusoids of plasmid DNA which has a gyration radius of approximately 0.1 μm.[35,36] Preliminary results using fluorescence-labeled DNA suggest that the increased osmotic and hydrostatic pressure was required for movement of the DNA out of the sinusoids and to the hepatocytes (data not shown).

We had previously proposed that plasmid DNA was taken up by muscle cells by an active process.[37] This was based upon the expression of reporter genes distant from the site of intramuscular injection *in vivo* and the ability of mature cultures of primary rat myotubes to express naked plasmid DNA. The high efficiency of hepatocyte expression from the intraportal route argues that some types of mammalian cells indeed have an intrinsic ability to take up naked plasmid DNA. The use of an intravascular route of administration provides irrefutable evidence that a direct mechanical effect from a needle is not required for naked DNA uptake and expression as was previously suggested for hepatocyte uptake.[8,9] However, we cannot exclude the possibility that the increased osmotic or hydrostatic pressure not only enabled DNA exit from sinusoids but was also required for or enhanced cellular uptake and expression of DNA. Just as naked DNA did not express luciferase in simple cultures of fibroblasts or C2-C12 myotubes,[37] HepG2 cells in culture also did not express naked DNA even under hyperosmotic conditions (data not shown). Studies of plasmid DNA uptake by mature, confluent cultures of primary hepatocytes may provide insight into the mechanism. It may be that certain types of cells within tissues *in situ* but not under routine culture conditions *in vitro* have an intrinsic ability to take up naked DNA. An effective route for *in vivo* administration of the DNA may be critical to reveal this ability. Tissue disruption and cell isolation may modify the cell inhibiting the cellular uptake of naked DNA.

The hGH reporter gene system was used to explore the ability of this gene transfer method to supply proteins to the systemic circulation. Two days after intraportal injection of a hGH expression plasmid, mean hGH serum levels in the untreated and dexamethasone-treated groups combined were 65 ± 26 ng/ml ($n = 7$). In humans, normal pulsatile levels of GH peak at approximately 20 ng/ml above baseline values of approximately 1 ng/ml and can attain concentrations of 10–180 ng/ml after growth hormone releasing hormone (GHRH) stimulation.[38–41] The half-life of hGH is approximately 20 min in humans and 4.5 min in mice; hence, these serum levels could translate into much higher levels for more stable proteins.[38,42] These results demonstrate that the intraportal naked DNA technique could be used to produce therapeutic levels of a circulating serum protein.

At 4 days after injection, hGH levels acutely dropped off to approximately 10 ng/ml in both untreated or dexamethasone-treated animals (Figure 3). The reason for this rapid decrease is unclear. A similar early drop-off in expression in the liver has been observed with adenoviral vectors.[43,44] At longer times in both untreated and dexamethasone-treated animals, hGH expression continued to decrease. Unstable hepatic expression of luciferase was also observed (data not shown). In previous studies, unstable expression has been reported when the plasmid DNA was delivered to nonhepatectomized livers using polylysine complexes or intralobar injections of naked DNA.[8,9,20–22] Preliminary data indicate that more stable hGH expression is achieved in animals that receive both cyclosporin A and dexamethasone. If the immune system is suppressed, then the slow mitotic rate of hepatocytes[45,46] may enable prolongation of plasmid expression just as the post-mitotic state of myofibers enables stable expression from naked DNA in muscle.[47] Stable plasmid expression from polylysine complexes has also been reported in the liver following partial hepatectomy[20–22] and in nonhepatectomized livers with complexes containing high ratios of poly-L-lysine to DNA.[48]

Further studies are needed to determine whether the intraportal delivery of naked DNA is of practical use for the treatment of inborn errors of metabolism and other disorders such as the hemophilias. The efficiency of the expression of various genes needs to be determined in larger animals. Often gene-transfer techniques efficient in mice are less so in large animals.[49,50] Preliminary results in rats are encouraging (data not shown). Also, the similar efficiencies of direct intralobar DNA injection in rats and cats suggest the high efficiency of intraportal delivery will also extrapolate to larger animals.[8,9] Furthermore, the larger size of the vascular systems in these bigger animals will enable better and easier surgical techniques to improve its efficiency and safety. Necrosis observed in approximately 10% of the sections may have been due to the increased hydrostatic or osmotic pressure.[51] Further optimization of the procedure in larger animals should enable efficient gene transfer without necrosis given the lack of hepatocellular damage in some livers with high β-galactosidase expression.

Repetitive administrations could be performed using catheters and noninvasive surgical techniques. The inability of repetitive intramuscular injections of naked DNA to induce anti-DNA antibodies in nonhuman primates suggests that repetitive intraportal administrations will be possible.[49,50] The preparation of sufficient quantities of naked plasmid DNA for human trials is feasible and less expensive than other types of nonviral and viral vectors.[49,50] It also avoids the potential problem of replication-competent viruses that appear in the preparation of viral vectors.

In conclusion, this is the first report of efficient expression of naked DNA in parenchymal cells following its intravascular delivery. Unprecedented levels of foreign gene expression have been achieved in the liver using a naked DNA approach.

Materials and methods

Intraportal injections of plasmid DNA

After the livers of 25 g, 6-week-old ICR mice were exposed through a ventral midline incision, solutions containing plasmid DNA were manually injected over approximately 30 s into the portal vein using a 30-gauge, 1/2 -inch needle and 1-ml syringe. In some animals, a

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Expression of Naked Plasmid DNA Injected into the Afferent and Efferent Vessels of Rodent and Dog Livers

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ABSTRACT

A variety of reporter genes within plasmid constructs were injected into the afferent and efferent vessels of the liver in mice, rats, and dogs. Efficient plasmid expression was obtained following delivery via the portal vein, the hepatic vein, and the bile duct. The use of hyperosmotic injection solutions and occlusion of the blood outflow from the liver substantially increased the expression levels. Combining these surgical approaches with improved plasmid vectors enabled uncommonly high levels of foreign gene expression in which over 15 μg of luciferase protein/liver was produced in mice and over 50 μg in rats. Equally high levels of β-galactosidase (β-Gal) expression were obtained, in that over 5% of the hepatocytes had intense blue staining. Expression of luciferase or β-Gal was evenly distributed in hepatocytes throughout the entire liver when either of the three routes were injected. Peri-acinar hepatocytes were preferentially transfected when the portal vein was injected in rats. These levels of foreign gene expression are among the highest levels obtained with nonviral vectors. Repetitive plasmid administration through the bile duct led to successive events of foreign gene expression. The integration of these findings into laboratory and clinical protocols is discussed.

OVERVIEW SUMMARY

Previously, we have shown that the intraportal injection of plasmid DNA in hypertonic solution leads to high levels of hepatocyte transfection in mice. This report found efficient expression following the retrograde injection of plasmid DNA into the hepatic vein and bile duct. For mice, the retrograde injection of naked DNA into the hepatic vein with occlusion of the portal vein or retrograde injection into the bile duct exhibits up to 10% of transfected hepatocytes. The same results were obtained in rats and qualitatively similar preliminary results were obtained in dogs. A protocol involving catheterization of the bile duct enabled repeat gene injections without additional surgery. The high efficiency of expression in larger animals and the use of relatively accessible vessels such as the hepatic vein or bile duct demonstrates the potential clinical utility of these gene transfer protocols.

INTRODUCTION

WE HAVE PREVIOUSLY SHOWN that plasmid DNA (pDNA) as expressed in the murine liver after injection into the portal vein, the organ's major afferent vessel (Budker *et al.*, 1996). Highly efficient expression required the use of hypertonic solutions containing heparin and restricting outflow from the liver by occlusion of the hepatic vein. We hypothesized that these conditions increased the openings of the siusoidal fenestrae beyond their normal size of ~100 nm to enable pDNA with an hydrodynamic radius of ~100 nm to pass through (Fraser *et al.*, 1980; Fisherman and Patterson, 1996). The efficiency of luciferase expression *in vivo* was similar to that obtained with the transfection of HepG2 cells *in vitro* using cationic lipids as a delivery system. Injection of a human growth hormone (hGH) expression vector resulted in over 50 ng/ml of hGH and injection of β-galactosidase (β-Gal) expression vector resulted in blue staining in ~1% of hepatocytes throughout the mouse liv-

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ers. These levels of expression from naked pDNA were unprecedented.

The present study explores the use of more accessible vessels such as the hepatic vein and the bile duct for delivering the naked pDNA in mice. Efficient gene expression was obtained using these efferent delivery routes. Occlusion of other vessels to restrict outflow of the injection solutions enhanced, but was not critical for, efficient expression. Repetitive injections into the bile duct were also accomplished. Preliminary results are also presented in larger animals—the rat and dog. The incorporation of these findings into laboratory and clinical protocols is discussed.

MATERIALS AND METHODS

Plasmid constructs

The pCILuc plasmid expresses a cytoplasmic luciferase from the human cytomegalovirus (CMV) immediately early (hCMV IE) promoter. It was constructed by inserting the luc+ gene, an *Nhe* I–*Eco* RI luc+ fragment from pSPLuc+ (Promega, Madison, WI), into the pCI expression vector (Promega). pCILux expresses peroxisomal luciferase under control of the hCMV IE promoter. It was constructed by inserting the luciferase gene (*Hind* III–*Bam* HI fragment from pBlueCMVLux) into the *Sma* I site of pCI. pCILacZ was constructed by placing the *Escherichia coli* LacZ gene (*Pst* I–*Apa* I fragment pBS-RSV-LacZ) into the pCI vector (*Sma* I site). The pCMVGH construct was previously described (Andree *et al.*, 1994).

Injection methods

Plasmid delivery into the hepatic vessels was performed in 6-week-old ICR mice, 2.5- to 6.5-month-old, 200- to 300-gram Sprague-Dawley rats, and beagle dogs. Ventral midline incisions were performed to expose the liver and the associated vessels. The mice were anesthetized with intramuscular injections of 1.000 μg of ketamine-HCl (Parke-Davis, Morris Plains, NJ) and methoxyflurane (Pitman-Moore, Mudelein, IL), which was administered by inhalation as needed. The rats were anesthetized with ether and the dogs were anesthetized with halothane by inhalation. The pDNA was injected in solutions containing 2.5 units/ml of heparin (Lypho-Med, Inc., Chicago, IL) (Qian *et al.* 1991) and either normal saline (0.9% NaCl) or 15% mannitol in normal saline (Sigma Chemical Co., St. Louis,

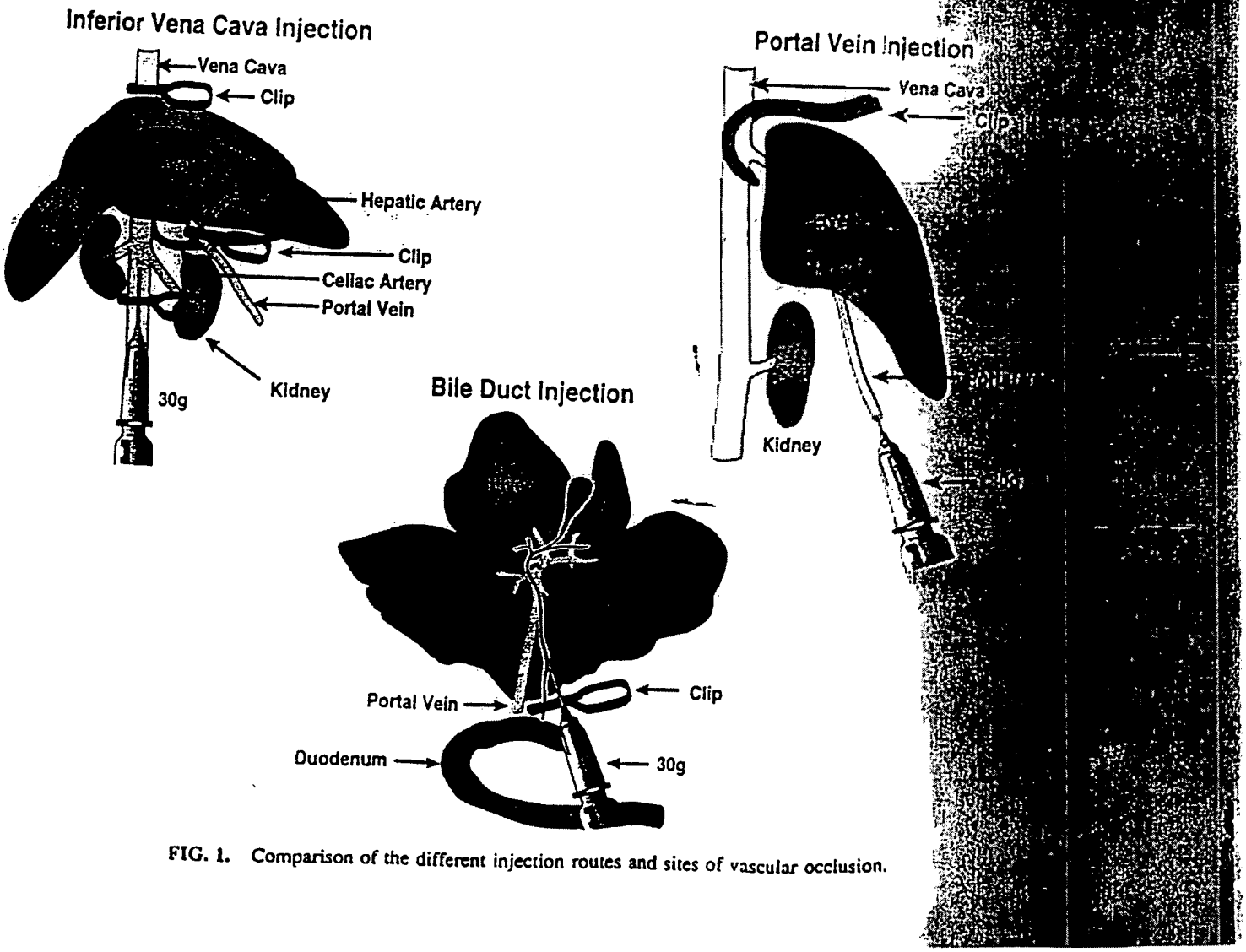

FIG. 1. Comparison of the different injection routes and sites of vascular occlusion.

MO). All animals received humane care in compliance with institutional (IACUC) guidelines.

Figure 1 illustrates the three routes of injection [portal vein, hepatic vein via an occluded inferior vena cava (IVC), and bile duct] and their associated outflow occlusions. In mice, the intraportal injections were performed as previously described (Budker *et al.*, 1996). A total of 100 μg of pDNA in 1 ml was manually injected over ~30 sec using a 30-gauge, 1/2-inch needle and 1-ml syringe without occluding the portal vein upstream from the point of injection. In some animals, a 5 × 1 mm, Kleinert-Kutz microvessel clip (Edward Weck, Inc., Research Triangle Park, NC) was applied during the injection at the junction of the hepatic vein and caudal vena cava.

DNA was delivered in mice to the hepatic vein via an occluded IVC. Clamps (6 × 1-mm, Kleinert-Kutz curved microvessel clip (Edward Weck, Inc., Research Triangle Park, NC)) were applied downstream (toward the heart) of the hepatic vein and upstream (towards the legs) of the hepatic and renal veins. Injections were done upstream of the hepatic vein. In some of the injections, the portal vein and hepatic artery were clamped using 6 × 1 mm, Kleinert-Kutz curved microvessel clips. The IVC mouse injections were also performed with 100 μg of pDNA in 1 ml that was manually injected over ~30 sec with a 30-gauge, 1/2-inch needle and 1-ml syringe.

The bile duct injections in mice were performed using manual injections with a 30-gauge, 1/2-inch needle and 1-ml syringe. A 5 × 1-mm, Kleinert-Kutz microvessel clip was used to occlude the bile duct downstream from the point of injection to prevent flow to the duodenum and away from the liver. The gallbladder inlet was not occluded. In some of the bile duct injections, the junction of the hepatic vein and caudal vena cava was clamped as above. In yet other injections, the portal vein and hepatic artery were clamped in addition to the occlusion of the hepatic vein. Survivability for all three injection routes in mice was almost 100%.

In mice, repetitive injections into the bile duct were done by placing a polyethylene tube (I.D. 0.28 mm, O.D. 0.61 mm; Intramedic Clay Adams Brand, Becton Dickinson Co., Sparks, MD) catheter into the bile duct after making a hole with a 27-gauge needle. The tubing was secured by a suture around the bile duct and tubing, thereby occluding the bile duct. The other end of the tubing was placed outside the skin of the animal's back so that surgery was not required for repeat injections. No blood vessel occlusions were done for these repetitive administrations. After completion of the studies, anatomical examination indicated that the catheter remained in the bile duct.

In rats, the intraportal, IVC, and bile duct injections were done as in mice but with the following modifications. The injections were done through a 25-gauge butterfly needle using a peristaltic pump (Preston varistaltic power pump, Manostat Corp., New York, NY) over 1 or 3 min. The downstream IVC clamps in the IVC injections were done downstream of the kidneys. For the portal vein injections, the portal vein and hepatic artery were clamped. The outflow through the hepatic vein was restricted in some animals by clamping the upstream and downstream IVC. In some animals the livers were first flushed with normal saline prior to DNA injection. Rat bile duct injections were done the same as mice. The rate does not have a gallbladder.

In some of the rat portal vein injections, a 25-gauge needle connected to a pressure gauge (Gilson Medical Electronics, Model ICT-11 Unigraph), was inserted into the liver parenchyma to determine the peak pressure within the liver during the injections. The stastical relationship between pressure and luciferase was done using Spearman's rank correlation. A smoothing spline regression model describing the relationship between log luciferase and pressure was estimated using generalized additive model methodology (Hastie, 1992). Akaike's information criteria (Akaike, 1973) indicated that a smoothing spline with 2 degrees of freedom resulted in the best fit over all integer degrees of freedom between 0 and 5.

The injections in dogs were done as in rats except that an 18-gauge, 2-inch angiocath (Becton Dickinson, San Jose, CA) was used. All dogs except dog #1 were females. Table 1 indicates the injection conditions. For the bile duct injections, a suture was applied to occlude the bile duct transiently downstream from the point of injection. A DeBakey multipurpose vascular clamp was applied to the cystic duct during injection to prevent the injected solution from entering the gallbladder. In dogs, the DNA was pCILux.

Protein assays

The luciferase assays were done as previously reported (Wolff *et al.*, 1990). One day after pCILuc injections, the animals were sacrificed and the rodent livers were divided into six sections composed of right lateral lobe, caudate lobe, two pieces of median lobe, and two pieces of left lateral lobe. For each of the six pieces, 0.7 ml of lysis buffer [0.1% Triton X-100, 0.1 *M* potassium phosphate, 1 m*M* dithiothreitol (DTT) pH 7.8] was used for mice and 4 ml of lysis buffer was used for rat liver. For the dog livers, approximately 10% of each lobe was divided into 5–20 pieces and placed into 2 ml of lysis buffer. The samples were homogenized using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT) and centrifuged at 4,000 rpm for 10 min at 4°C. Twenty microliters of the supernatant was analyzed for luciferase activity. Relative light units (RLU) were converted to picograms of luciferase using standards from Analytic Luminescence Laboratories (ALL, San Diego, CA). The amount of luciferase protein (pg) was calculated using a standard curve in which luciferase protein (pg) = $5.1 \times 10^{-5} \times$ RLU + 3.683 ($r^2 = 0.992$).

Ten-micrometer-thick tissue sections were stained for β-galactosidase (β-Gal) expression as previously described using 1- to 4-hr X-Gal incubations (Budker *et al.*, 1996). Hematoxylin was used for the counterstain but the alkaline step was omitted so that the hematoxylin stain remained red. The percent of blue-stained cells in the liver sections was determined by counting ~3,000 cells in three sections and averaging.

Blood obtained from the retro-orbit sinus was analyzed for serum concentration of hGH using the radioimmune assay (RIA) HGH-TGES 100T kit from Nichols Institute (San Juan Capistrano, CA). Serum ALT and GGT levels were done using EKTACHEM DT slides and a KODAK EKTACHEM DT 60 ANALYZER as recommended by the manufacturer (Kodak, Rochester, NY).

The statistical significance of differences between the means from the two independent samples of growth hormone data was tested with the t statistic. The statistical significance of differences for the luciferase data was tested with one-way analysis

of variance (ANOVA) using the SAS generalized linear model procedure because more than two independent samples were involved.

RESULTS

Mice luciferase experiments

Our previous studies used the pBS.CMVLux plasmid (expressing the peroxisomal native luciferase from the CMV promoter) for evaluating the optimal conditions for naked pDNA expressing following intraportal injection. These optimal conditions were intraportal injections of 100 μg of pDNA in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. The injections were done over 30 sec with the hepatic vein and IVC occluded. In this study, 100 μg of pCILuc (expressing a cytoplasmic modified luciferase from a CMV promoter with a chimeric intron) injected under similar conditions yielded a mean total luciferase protein/liver of 3.73 μg/liver (Fig. 2A, PV+CL), approximately 30 times greater than that obtained with pBS.CMVLux. Part of this increase could be attributed to greater operator experience with these injection techniques. Injections with pCILuc under these conditions without clamping the hepatic vein yielded approximately 750-fold less luciferase (Fig. 2A, PV-CL).

The hepatic veins (via the IVC) of another set of mice were injected with 100 μg of pCILuc in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. A mean total luciferase protein/liver of 17.34 μg/liver (Fig. 2A, IVC+CL) was obtained when the portal vein clamped as compared to a mean total luciferase protein/liver of 2.83 μg/liver (Fig. 2A, IVC-CL) without occluding the portal vein.

Similar results were also obtained when bile ducts were injected with 100 μg of pCILuc in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. A mean total luciferase protein/liver of 15.39 μg/liver (Fig. 2A, BD+CL) was obtained when the hepatic vein was clamped as compared to a mean total luciferase protein/liver of 1.33 μg/liver (Fig. 2A, BD-CL) without occluding the hepatic vein. If mannitol was omitted, then the bile duct injections without clamping any blood vessels yielded approximately 15-fold less luciferase (0.086 μg/liver $\pm$ 0.06, $n = 5$). Clamping the hepatic artery and portal vein in addition to the hepatic vein did not improve expression beyond what was obtained when only the hepatic vein was clamped (data not shown). For all three injection routes, outflow occlusion significantly ($p < 0.05$) increased luciferase expression.

Serum ALT and GGT assays were performed on mice 1 and 8 days after each of the above injections with pCILuc (4 mice for each condition). No increases in GGT were observed after any of the injections, including the bile duct injections. Serum ALT levels increased to 200–400 units/liter 1 day after portal vein and bile duct injections. One day after IVC injections, serum ALT levels increased to ~1,500 units/liter in half of the mice but was only ~250 units/liter in the other half. By 18 days after injection, serum ALT levels decreased to baseline levels in all animals. For positive control purposes, a nonlethal intraperitoneal injection of 40 μl of 50% carbon tetrachloride in mineral oil was performed. An average of 25,900 units/liter

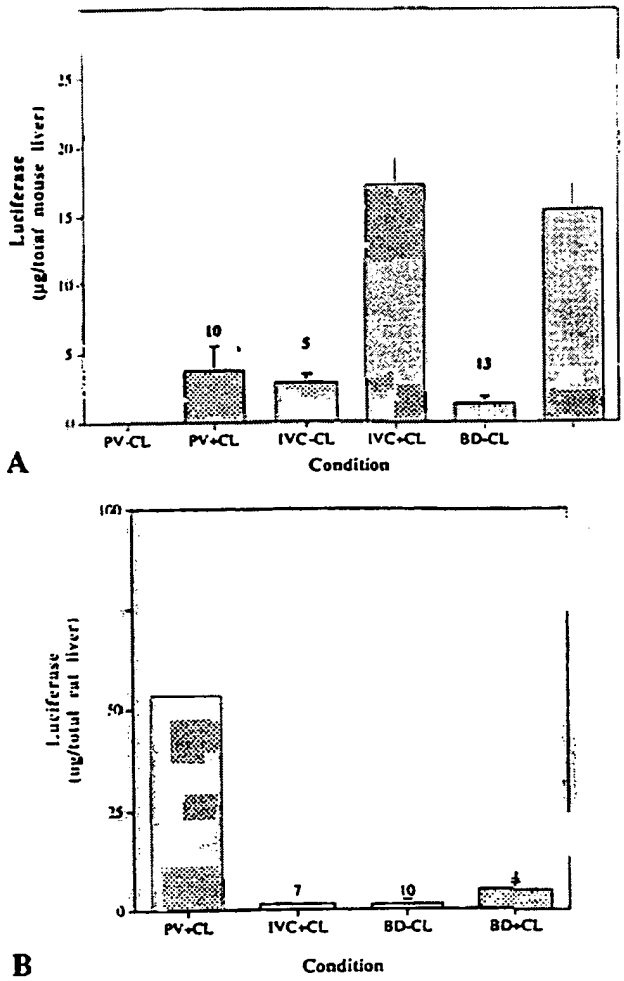

FIG. 2. Comparison of total luciferase expression in mice (A) and rats (B) injected with pCILuc under various conditions. The condition abbreviations signify the following: PV − CL, portal vein injections without clamping; PV + CL, portal vein injections with clamping the hepatic vein; IVC − CL, IVC injections without clamping; IVC + CL, IVC injections with clamping the portal vein and hepatic artery; BD − CL, bile duct injections without clamping; BD + CL, bile duct injections with clamping the hepatic vein. Numbers above the bars indicate the number of mice. T bars indicate the standard error.

($n = 4$) was observed 1 day after injection. This comparison indicates that the DNA injection procedure caused minimal and transient liver toxicity.

Rat and dog luciferase experiments

Similar injections into the portal vein, IVC (to the hepatic vein), and bile duct were done in rats (Fig. 2B). For the portal vein injections, the injection volumes were increased 15 times over that used in mice because the rat livers are ~15 times larger than mouse livers. The amount of pCILuc was only increased ~7.5 times because the use of more pCILuc did not result in significantly more luciferase expression (data not shown). One day after 750 μg of pCILuc in 15 ml of 15% man-

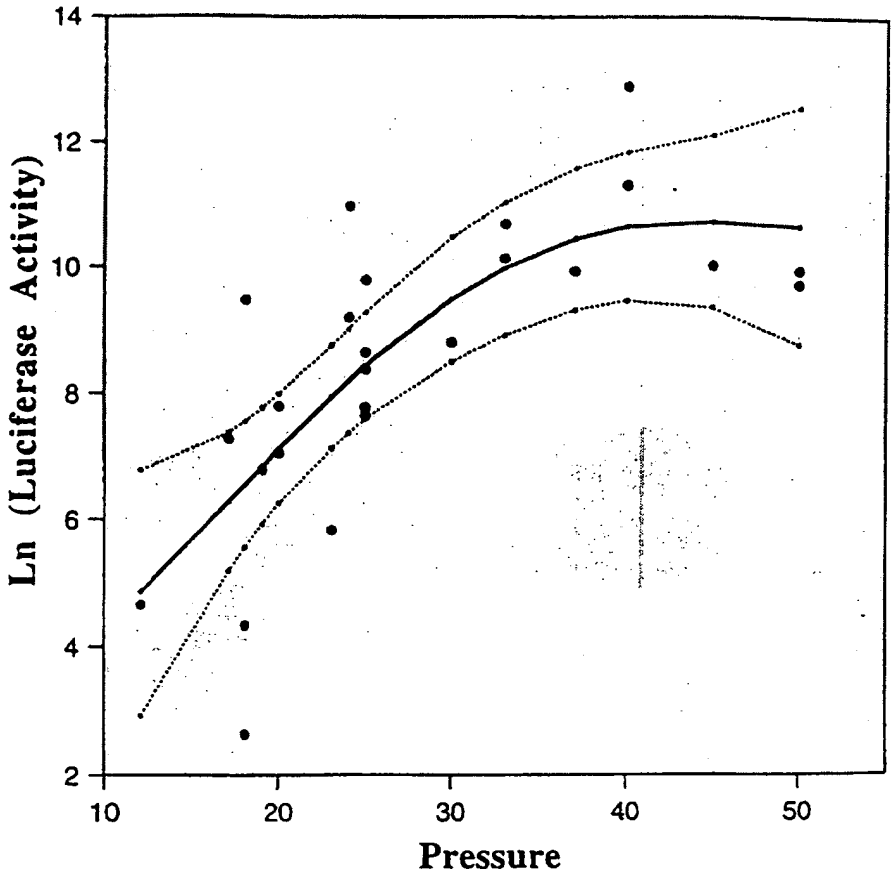

FIG. 3. Scatterplot of the log(e) of luciferase (ng protein) versus peak, intrahepatic, parenchymal pressure (mmHg). The solid line indicates the best fit. Dotted lines indicate 95% pointwise prediction intervals.

nitol and 2.5 units of heparin/ml in normal saline solution were injected into the portal vein while occluding the hepatic vein, an average of 53.5 μg of luciferase/liver was obtained (Fig. 2B, PV+CL). The efficiency of gene transfer in the rat was compared to that in mice in two ways. In terms of efficiency as defined by the nanograms of luciferase per milligram of tissue weight, the levels of expression for portal injections were 3.6 μg of luciferase/gram of tissue in rats as compared to 3.7 μg of luciferase/gram of tissue in mice. Alternatively, in terms of efficiency as defined by the nanograms of luciferase per microgram of pDNA delivered, the efficiencies of expression for portal injections were 71 ng of luciferase/μg of DNA in rats as compared to 37 ng of luciferase/μg of DNA in mice.

Less, but still substantial, luciferase expression was obtained when the injections of pCILuc were done into the efferent vessels of rats such as the IVC or bile duct (Fig. 2B). Injections of 750 μg of pCILuc in 15 ml into the hepatic vein (via the IVC) while occluding the portal vein yielded an average of 1.5 μg of luciferase/liver. Injections of 750 μg of pCILuc in 5–8 ml into the bile duct with outflow obstruction yielded an average of 4.9 μg of luciferase/liver.

Parenchymal pressures of 12–50 mmHg were measured in 23 rat livers during the injection of 750 μg pCILuc in 15 or 20 ml into the portal vein while occluding the IVC (Fig. 3). Spearman's rank correlation between pressure and luciferase expression was 0.76 (two-side p-value <0.001) indicating that pressure and luciferase were significantly positively associated. It appeared that pressures of over 40 mmHg did not result in increased expression. The necessity of the nonlinear component over and above a simple linear fit was verified by an approximate full versus reduced F-test (p value = 0.014), indicating that the observed plateau effect is real. Examination of both the residual quantile-quantile and the residual versus prediction plots further reveals that there are no serious violations of the regression model assumptions and therefore the regression models and p values are valid (Fisher and van Belle, 1993).

Preliminary experiments explored the ability of naked pCILux (not pCILuc) to be expressed in dogs (Table 1). In mice, in the case of intraportal injection, pCILux provided three times lower expression than pCILuc (data not shown). In 5 dogs, various amounts of DNA were injected into either the bile duct with or without blocking outflow by occluding the IVC. In 1 animal, the DNA was injected into the IVC without any outflow blockage. All the dogs survived the procedure except animals that had the IVC occluded recovered more slowly postoperatively. The animals were sacrificed 1 day after the injections and dozens of tissue samples from each liver lobe were analyzed for luciferase. The luciferase expression was evenly distributed over all the lobes in each liver except in one lobe of 1 dog. Routine histological analysis in dog #5 (Table

TABLE LUCIFERASE EXPRESSION FOLLOWING THE INJECTION OF pCILUX INTO THE BILE DUCT OR IVC OF DOGS

Dog#	Total LUX (ng)	Liver wt (g)	Dog wt (kg)	DNA amount (mg)	Vessel injected	Out flow block	Inject volume (ml)	Plasmid conc. (μg/ml)	Inject rate (ml/min)
		290.7		10	Bile duct	no	300	33	75
2		357.9		10	Bile duct	no	300	33	60
3		244.2		14	Bile duct	yes	280	50	70
4		430.1		30	Vena cava	no	600	50	120
5		310.2		20	Bile duct	yes	400	50	100
6		307.1		20	Bile duct	no	200	100	66

l) indicated that the tissue architecture was substantially disrupted, suggesting that the injection conditions were not optimal. Decreasing the volume of injection in dog #6 to 200 ml resulted in the best expression (Table 1).

Mice and rat β-Gal results

The β-Gal expression vector was used to determine the percent and type of cells that were transfected (Fig. 4). As previously noted for portal vein injections (Budker *et al.*, 1996), the vast majority of the blue-stained cells appeared to be hepatocytes on morphological grounds but a few appeared to be endothelial or other types of cells. A preponderance of hepatocytes were also stained blue after the bile duct or IVC injections in mice or rats (Fig. 4). No hepatocytes were stained blue after similar injections of pCILuc.

In mice, all three injection procedures resulted in 5–10% of the hepatocytes expressing β-Gal (Fig. 4A–C). In rats, the portal vein injections gave the highest percentage of β-gal-positive cells in which 7% of hepatocytes were positive (Fig. 4D). Much fewer β-Gal-positive cells were noted in rats injected in the IVC (Fig. 4E) or bile duct (data not shown). In some animals, liver cell damage was evident in less than 5% of the cells. Of note in the rat livers injected into the portal vein, almost all of the positively stained cells were peri-acinar with few positive cells around the central vein (Fig. 4D).

Repeat bile duct injections

The bile ducts of mice were cannulated and 100 μg of pCMVhGH in 1 ml of 15% mannitol in normal saline were injected once a week (Fig. 5). Serum levels of hGH increased 1 day after the first injection and then decreased to background levels by 7 days after injection. One day after the second injection, hGH levels again increased and then were back to background levels by 7 days after the second injection. Only minimal increases in hGH levels occurred after the third injection. Mice that had the highest levels after the first injection had the lowest levels after the second injection (mice 3 and 6) and vice versa (mice 1, 2, and 4). Luciferase levels were significantly different between day 1 and day 7 ($p < 0.02$) but were not significantly different between day 9 and day 14 because of the large variability after the second injection. Control mice that ceived no injections or injections with a luciferase pDNA had mean hGH levels of 0.3 ± 0.1 ng/ml.

In another set of animals (4 mice), the bile duct injections were repeated four times with pCMVhGH and then pCILuc was injected. The first three pCMVhGH injections led to similar increases in hGH serum levels as in Fig. 5. Although there were only minimal raises in hGH serum levels following the fourth injection, injection of pCILuc yielded an average of 29.2 ng/liver (±7.1, $n = 3$). The liver in 1 of the 4 mice was grossly yellow and scarred as a result of the bile duct ligation and did not express any luciferase.

DISCUSSION

This report extends the findings of the previous study showing pDNA expression following afferent intraportal delivery and demonstrates efficient plasmid expression following delivery via efferent vessels such as the hepatic vein or bile duct. Expression of luciferase or β-Gal was evenly distributed throughout the entire liver when either of the three vessels was injected. Combining these surgical approaches with improved plasmid vectors enabled uncommonly high levels of foreign gene expression in which over 15 μg of luciferase protein/liver was produced in mice and over 50 μg in rats (Figs. 2 and 3). Equally high levels of β-Gal expression were obtained in that 5–10% of the hepatocytes had intense blue staining (Fig. 4). These levels of foreign gene expression are among the highest levels obtained with nonviral vectors.

Using the portal vein administration route, occlusion of the outflow is critical for expression. Outflow occlusion increases the expression with the efferent administrative routes, but substantial amounts of expression were obtained even when the hepatic vein was not blocked. Most likely the natural direction of blood flow provides a sufficient impetus to retard the egress of injection fluid and raise the hydrostatic pressure. The use of these efferent vessels simplifies the administration for potential human applications because they are easier to access by noninvasive methods. If no occlusion is used, then only one vessel has to be reached. These efferent routes should also be considered for the administration of viral and nonviral vectors as has been done with the delivery of adenoviral vectors into the bile duct (Yang *et al.*, 1993; Vrancken Peeters *et al.*, 1996a,b).

The mechanism of pDNA uptake is not known but may involve native cellular uptake processes (Budker *et al.*, 1996). It is of interest that high levels of luciferase expression could occasionally be obtained when the DNA was injected into the bile duct in small volumes of isotonic solutions without occluding the IVC. Increased osmolar and hydrostatic pressure may not

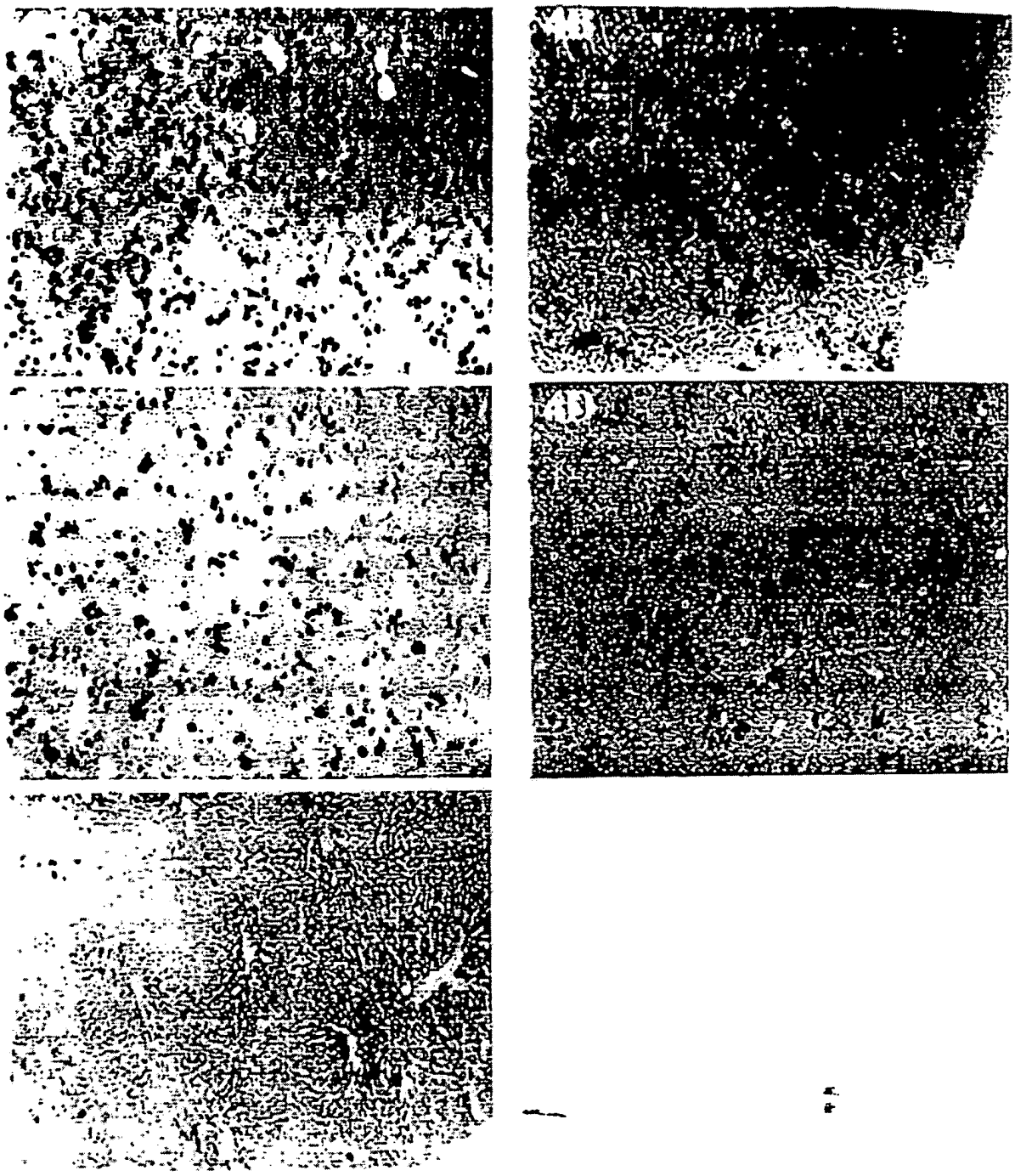

FIG. 4. Histochemical analysis of β-Gal expression in livers after injection of pCILacZ into: mouse portal vein with the hepatic vein clamped (A), mouse IVC with the portal vein and hepatic artery clamped (B), mouse bile duct with the hepatic vein clamped (C), rat portal vein with the IVC clamped (D), and rat IVC with the portal vein and hepatic artery clamped (E). Mouse injections were done using 100 μg of pCILacZ in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution whereas the rat injections were done using ~50 μg of pCILacZ in 15 ml of the same solution. Magnifications, 160× (A and B); 100× (C).

be critical for uptake of the pDNA by hepatocytes, as they are not in muscle cells (Wolff *et al.*, 1990, 1991, 1992a). This would suggest that the mechanism of pDNA uptake may in fact involve endogenous cellular pathways. Increased hydrostatic and osmotic pressures may raise expression by enhancing these cellular internalization processes (Haussinger, 1996).

The raised pressures could also increase the delivery of the pDNA to the hepatocyte surface not only for the blood vessel

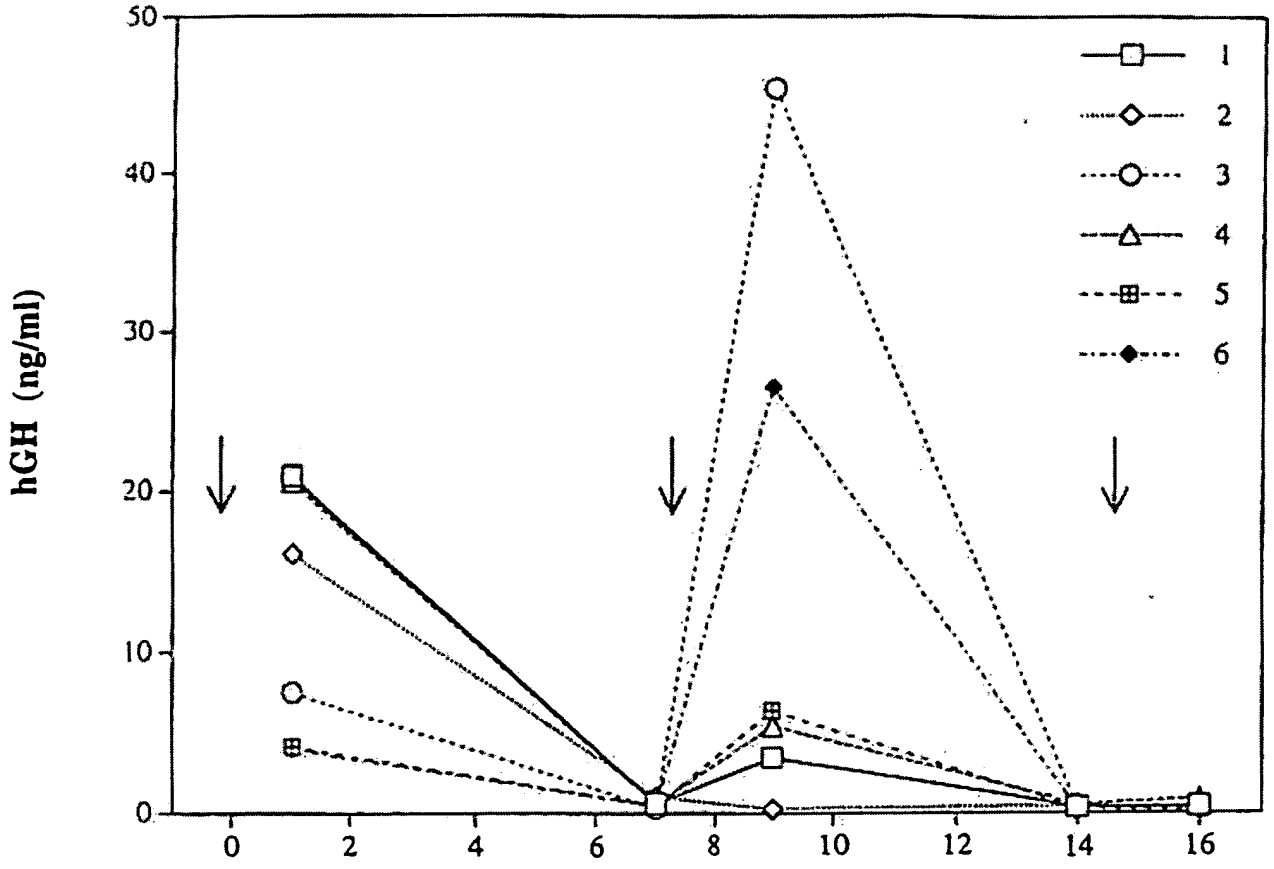

FIG. 5. Human growth hormone (hGH) levels following the repetitive administration (arrows indicates timing) of 100 μg of pCMVhGH into the bile duct of mice via a cannula. Numbers identify individual mice.

pression of the immune system enables more persistent expression. In post-mitotic myofibers, plasmids can persist extrachromosomally and express for at least 2 years, presumably because the pDNA is not being lost as result of cell division (Wolff *et al.*, 1992b; Herweijer *et al.*, 1995). Quite possibly pDNA would be lost slowly in hepatocytes, which have a half-life of up to a year in rodents and humans (Leffert *et al.*, 1988; Webber *et al.*, 1994). If so, the liver-based genetic disorders such as hemophilia could be treated by injections every 6 months. The bile duct could be accessed repeatedly by upper gastrointestinal endoscopy. Similarly, the hepatic vein could be noninvasively accessed via peripheral or central veins. In addition, gene transfer could be delivered to newborns via the umbilical cord vessels to get them over a newborn metabolic crisis, as occurs in the organic acidurias and the urea cycle defects.

Often gene transfer techniques that work in mice do not work in larger animals. Our results demonstrate than the technique works in rats that are approximately 10-fold bigger that mice. The dog results indicate that the liver of larger nonrodent mammal can express naked pDNA. Although substantial levels of luciferase activity were obtained, further optimization of the injection conditions is required to increase the efficiency of expression so that they are comparable to those in rodents. The studies to determine the relationship between intraparenchymal pressure and luciferase expression in rats are a first step toward this goal (Fig. 3). Minimal liver cell damage occurred in the rodents as evident by serum chemistries and histology but the injections were more disruptive to the hepatocytes in dogs. Presumably, the key factor is the efficient delivery of the pDNA to the hepatocyte surface with minimal cellular or tissue disruption.

In the research laboratory, the described techniques will enable rodents to be used just as immortalized and primary liver cell cultures are now used for gene and cellular studies of liver function. The transfer of genes into cells in culture have been a critically important tool for deciphering the function of genes and for studying the effect of expressed proteins on cellular processes. Typically, the gene under study is placed within a plasmid vector and transiently transfected into the appropriate cell in culture. Isoforms and mutant forms of the gene under study can be quickly placed into plasmid expression vectors and studied. Our findings indicate that a similar plasmid-based approach could be used to study the effects of gene function in hepatocytes *in situ*. Given that the high levels of expression are transient in this system, it would be best if these effects occurred within a few days. The use of pDNA avoids the laborious steps necessary for the production of viral vectors or generation of transgenic mice and thereby enables many different genes and their related mutated forms to be quickly studied. It will permit the mechanism of gene expression and their effects on liver function to be expeditiously probed within the context of a complete mammalian organism.

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